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Fertel R. and Weiss, B., Methods in Enzymol. Vol LV  
II, Bioluminescence and Chemiluminescence,  
DeLuca, M.A. (Ed.) 94-106 (1978)

FILE COPY

*Methods in Enzymology*

*Volume LVII*

*Bioluminescence and  
Chemiluminescence*

EDITED BY

*Marlene A. DeLuca*

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LA JOLLA, CALIFORNIA

JAN 10 1979

SERIALS



ACADEMIC PRESS New York San Francisco London 1978

A Subsidiary of Harcourt Brace Jovanovich, Publishers

Ref. 9  
QP  
601  
.C733  
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ACADEMIC PRESS, INC.

111 Fifth Avenue, New York, New York 10003

*United Kingdom Edition published by*

ACADEMIC PRESS, INC. (LONDON) LTD.

24/28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data

Main entry under title:

Bioluminescence and chemiluminescence.

(Methods in enzymology ; v. 57)

Includes bibliographical references and index.

I. Bioluminescence. 2. Chemiluminescence.

I. DeLuca, Marlene. II. Title.

QP601.M49 vol. 57 [QH641] 574.1'925'08s

[574.1'9125]

ISBN 0-12-181957-4

78-13442

PRINTED IN THE UNITED STATES OF AMERICA

## Table of Contents

CONTRIBUTORS TO VOLUME LVII . . . . .	ix
PREFACE . . . . .	xiii
VOLUMES IN SERIES . . . . .	xv

### Section I. Firefly Luciferase

1. Purification and Properties of Firefly Luciferase	MARLENE DeLUCA AND W. D. McELROY	3
2. Synthesis of Firefly Luciferin and Structural Analogues	LEMUEL J. BOWIE	15
3. Preparation of Partially Purified Firefly Luciferase Suitable for Coupled Assays	HANS N. RASMUSSEN	28
4. Firefly Luciferase Assay for ATP Production by Mitochondria	JOHN J. LEMASTERS AND CHARLES R. HACKENBROCK	36
5. Measurement of Photophosphorylation and ATP- ase Using Purified Firefly Luciferase	ARNE LUNDIN AND MARGARETA BALTSCHIEFFSKY	50
6. Determination of Creatine Kinase Isoenzymes in Human Serum by an Immunological Method Using Purified Firefly Luciferase	ARNE LUNDIN	56
7. Determination of Bacterial Content in Fluids	EMMETT W. CHAPPELLE, GRACE L. PICCIOLO, AND JODY W. DEMING	65
8. Biomass and Adenylate Energy Charge Determina- tion in Microbial Cell Extracts and Environmen- tal Samples	OSMUND HOLM-HANSEN AND DAVID M. KARL	73
9. Determination of GTP, GDP, and GMP in Cell and Tissue Extracts	DAVID M. KARL	85
10. Measurement of the Activity of Cyclic Nucleotide Phosphodiesterases with Firefly Luciferin- Luciferase Coupled Assay Systems	RICHARD FERTEL AND BENJAMIN WEISS	94
11. Rapid Microassay for the Calcium-Dependent Pro- tein Modulator of Cyclic Nucleotide Phospho- diesterase	JOHN C. MATTHEWS AND MILTON J. CORMIER	107
12. ATP-Labeled Ligands and Firefly Luciferase for Monitoring Specific Protein-Binding Reactions	ROBERT J. CARRICO, R. DON JOHNSON, AND ROBERT C. BOGUSLASKI	113

### Section II. Bacterial Luciferase

13. Bacterial Bioluminescence: An Overview	J. W. HASTINGS	125
14. Bacterial Luciferase: Assay, Purification, and Properties	J. W. HASTINGS, THOMAS O. BALDWIN, AND MIRIAM Z. NICOLI	135
15. Isolation, Identification, and Manipulation of Luminous Bacteria	KENNETH H. NEALSON	153
16. Isolation and Characterization of Luminescence System Mutants in Bacteria	THOMAS W. CLINE	166
17. Preparation of the Subunits of Bacterial Luciferase	SHIAO-CHUN TU	171
18. Preparation of Luciferases Containing Chemically Modified Subunits	EDWARD A. MEIGHEN	174
19. Quantitation of Malate, Oxaloacetate, and Malate Dehydrogenase	PHILIP E. STANLEY	181
20. Bioassay for Myristic Acid and Long-Chain Aldehydes	S. ULITZUR AND J. W. HASTINGS	189
21. The Luciferase Reduced Flavin-Hydroperoxide Intermediate	J. W. HASTINGS AND JAMES E. BECVAR	194
22. Bacterial Luciferase as a Generalized Substrate for the Assay of Proteases	THOMAS O. BALDWIN	198
23. Immobilization of Bacterial Luciferase and Oxidoreductase and Assays Using Immobilized Enzymes	EDWARD JABLONSKI AND MARLENE DELUCA	202
24. Quantitation of Picomole Amounts of NADH, NADPH, and FMN Using Bacterial Luciferase	PHILIP E. STANLEY	215
25. Luminous Bacteria as an Oxygen Indicator	B. CHANCE AND R. OSHINO	223
26. Purification of a Blue-Fluorescent Protein from the Bioluminescent Bacterium <i>Photobacterium phosphoreum</i>	JOHN LEE AND PRASAD KOKA	226

### Section III. *Renilla reniformis* Luciferase

27. Applications of <i>Renilla</i> Bioluminescence: An Introduction	MILTON J. CORMIER	237
28. A Bioluminescence Assay for PAP (3', 5'-Diphosphoadenosine) and PAPS (3'-Phosphoadenylyl Sulfate)	JAMES MICHAEL ANDERSON, KAZUO HORI, AND MILTON J. CORMIER	244
29. Protein-Protein Interactions as Measured by Bioluminescence Energy Transfer in <i>Renilla</i>	WILLIAM W. WARD AND MILTON J. CORMIER	257

---

**Section IV. Aequorin**

30. Introduction to the Bioluminescence of Medusae, FRANK H. JOHNSON AND  
with Special Reference to the Photoprotein OSAMU SHIMOMURA 271  
Aequorin
31. Practical Aspects of the Use of Aequorin as a Cal- JOHN R. BLINKS, PATRICK H.  
cium Indicator: Assay, Preparation, Microinjec- MATTINGLY, BRIAN R.  
tion, and Interpretation of Signals JEWELL, MENNO VAN  
LEEUVEN, GARY C.  
HARRER, AND DAVID G.  
ALLEN 292

**Section V. Cypridina**

32. Introduction to the *Cypridina* System FRANK H. JOHNSON AND  
OSAMU SHIMOMURA 331
33. *Cypridina* Luciferin and Luciferase FREDERICK I. TSUJI 364

**Section VI. Earthworm Bioluminescence**

34. Assaying Hydrogen Peroxide Using the Earth- MICHAEL G. MULKERRIN  
worm Bioluminescence System AND JOHN E. WAMPLER 375

**Section VII. *Pholas dactylus***

35. Purification and Properties of *Pholas dactylus*  
Luciferin and Luciferase A. M. MICHELSON 385

**Section VIII. Chemiluminescent Techniques**

36. The Chemiluminescence of Luminol and Related DAVID F. ROSWELL AND  
Hydrazides EMIL H. WHITE 409
37. Monitoring Specific Protein-Binding Reactions HARTMUT R. SCHROEDER,  
with Chemiluminescence ROBERT C. BOGUSLASKI,  
ROBERT J. CARRICO, AND  
ROBERT T. BUCKLER 424
38. Chemiluminescence Detection of Enzymically  
Generated Peroxide W. RUDOLF SEITZ 445
39. The Generation of Chemiluminescence (CL) by MICHAEL A. TRUSH,  
Phagocytic Cells MARK E. WILSON, AND  
KNOX VAN DYKE 462

40. Chemiluminescence from Electron-Transfer Processes	LARRY R. FAULKNER	494
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### Section IX. Instrumentation and Methods

41. Construction of Instrumentation for Bioluminescence and Chemiluminescence Assays	JAMES MICHAEL ANDERSON, GEORGE J. FAINI, AND JOHN E. WAMPLER	529
42. The Simultaneous Determination of Heat Changes and Light Production	NEAL LANGERMAN	540
43. Characteristics of Commercial Instruments and Reagents for Luminescent Assays	GRACE LEE PICCIOLO, JODY W. DEMING, DAVID A. NIBLEY, AND EMMETT W. CHAPPELLE	550
44. Excited States and Absolute Calibrations in Bioluminescence	H. H. SELIGER	560
AUTHOR INDEX . . . . .		601
SUBJECT INDEX . . . . .		616



extremely heat-stable protein, back production of ATP will occur ( $2 \text{ ADP} \xrightleftharpoons{\text{MK}} \text{AMP} + \text{ATP}$ ) following the heat deactivation of coupled Reactions (3) and (4). By maintaining relatively low concentrations of ATP [i.e., ADP after Reaction (1)] and of GK, the effects of MK contamination are eliminated. If sufficiently high levels of ADP are present within the GTP extracts ( $>200 \text{ ng of ADP ml}^{-1}$ ), a small amount of light will be produced, after sample injection, owing to MK activity contained within the crude luciferase preparations. The amount of light emitted is less than 1% of the activity resulting from an equimolar concentration of GTP; however, if necessary, this source of interference can be evaluated (and corrected for) by measuring the sum of the concentrations of ATP and ADP within each sample extract<sup>20</sup> and relating these values to the reactivity of standard ADP solutions.

#### Acknowledgments

The author expresses his appreciation to Dr. O. Holm-Hansen for comments, criticism, and encouragement offered during the course of this research and the preparation of this chapter. Dr. F. Azam and Ms. L. Campbell critically reviewed the original manuscript and offered helpful suggestions for improvement. The methodology described in this report was developed under ERDA contract EY-76-C-03-0010 P.A. 20.

### [10] Measurement of the Activity of Cyclic Nucleotide Phosphodiesterases with Firefly Luciferin-Luciferase Coupled Assay Systems

By RICHARD FERTEL and BENJAMIN WEISS

The procedures described below are designed to measure cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase (EC 3.1.4.17) by reaction sequences using firefly luciferin and luciferase in the final step. These assay systems are simple, sensitive, inexpensive, and reproducible.

Intracellular concentrations of adenosine 3',5'-cyclic monophosphate (cyclic AMP) and guanosine 3',5'-cyclic monophosphate (cyclic GMP) affect a number of biochemical and physiologic processes in the cell.<sup>1-6</sup> Accordingly, certain cell functions may be controlled by altering the cyclic nucleotide concentrations in that cell. One way to influence the concentration of the cyclic nucleotides is by activating or inhibiting cyclic nucleotide phosphodiesterases, which catalyze the hydrolysis of the cyclic nucleotides to their 5'-monophosphate analogs. For this reason, these enzymes have been the subject of a number of investigations,<sup>6</sup> and a variety of methods have been devised to measure their activity.<sup>7-10</sup> We have developed for these enzymes assay procedures based on the quantitative coupling of the product of the phosphodiesterase reaction [either adenosine 5'-monophosphate (5'-AMP) or guanosine 5'-monophosphate (5'-GMP)] to adenosine-5'-triphosphate [ATP].<sup>11,12</sup> The concentration of ATP in the reaction mixture is then determined by means of the firefly luciferin-luciferase reaction.<sup>13</sup>

## Principle of the Assay Systems

### *Cyclic-AMP Phosphodiesterase*

In this reaction sequence, the phosphodiesterase converts cyclic AMP to its degradation product, 5'-AMP. The 5'-AMP, in the presence of a very low concentration of ATP, which serves as a phosphate donor, is converted by the enzyme myokinase (EC 2.7.4.3) to adenosine-5'-diphosphate

<sup>1</sup> G. A. Robison, G. G. Nahas, and L. Triner, eds. *Ann. N. Y. Acad. Sci.* 185 (1971).

<sup>2</sup> P. Greengard, R. Paoletti, and G. A. Robison, eds. "Advances in Cyclic Nucleotide Research," Vol. 1, Raven, New York, 1972.

<sup>3</sup> P. Greengard and E. Costa, eds. *Adv. Biochem. Psychopharmacol.* 3 (1970).

<sup>4</sup> N. D. Goldberg, R. F. O'Dea, and M. K. Haddox, in "Advances in Cyclic Nucleotide Research" (P. Greengard and G. A. Robison, eds.), Vol. 2, p. 155. Raven, New York, 1973.

<sup>5</sup> B. Weiss, ed. "Cyclic Nucleotides in Disease." Univ. Park Press, Baltimore, Maryland, 1975.

<sup>6</sup> B. Weiss, and R. Fertel, *Adv. Pharmacol. Chemother.* 14, 189 (1977).

<sup>7</sup> R. W. Butcher and E. W. Sutherland, *J. Biol. Chem.* 237, 1244 (1962).

<sup>8</sup> W. J. Thompson and M. M. Appleman, *J. Biochem.* 10, 311 (1971).

<sup>9</sup> C. R. Filburn and J. Karn, *Anal. Biochem.* 52, 505 (1973).

<sup>10</sup> W. Y. Cheung, *Anal. Biochem.* 28, 182 (1969).

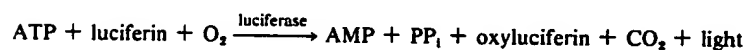
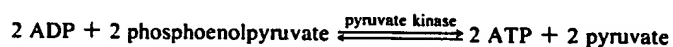
<sup>11</sup> B. Weiss, R. Lehne, and S. Strada, *Anal. Biochem.* 45, 222 (1972).

<sup>12</sup> R. Fertel and B. Weiss, *Anal. Biochem.* 59, 386 (1974).

<sup>13</sup> B. L. Strehler and J. R. Totter, *Arch. Biochem. Biophys.* 40, 28 (1952).

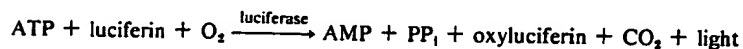
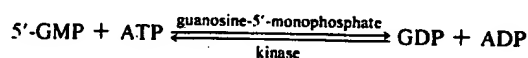
(ADP). ADP is then converted to ATP in the presence of phosphoenolpyruvate, which serves as a phosphate donor, and the enzyme pyruvate kinase (EC 2.7.1.40), which catalyzes the reaction.

Under the conditions specified below, the 5'-AMP is rapidly and completely converted to ATP, which is then measured using the firefly luciferin-luciferase reaction. The entire sequence is shown below:



#### *Cyclic-GMP Phosphodiesterase*

In this reaction sequence, the 5'-GMP formed as the product of the phosphodiesterase reaction alters the equilibrium of the reaction catalyzed by guanosine-5'-monophosphate kinase (EC 2.7.4.8). This leads to a decrease in ATP that is proportional to the concentration of 5'-GMP formed. The ATP concentration is then determined by reaction with firefly luciferin and luciferase. The reaction sequence is as follows:



#### **Reagents Used in the Assay Systems**

##### *Reagents for the Cyclic-AMP Phosphodiesterase Assay*

###### *Reagent A*

Glycylglycine buffer, 150 mM pH 8.0

Ammonium acetate, 75 mM

Magnesium chloride, 9 mM

Calcium chloride, 30  $\mu$ M  
Phosphoenolpyruvate, 0.78 mM  
Dithiothreitol, 15 mM  
ATP, 3 nM

Reactants in this reagent are all at 3 times their final concentration. The reagent is stable for several months at  $-4^{\circ}$ . On the day of the assay, 3% bovine serum albumin (BSA), myokinase, and pyruvate kinase, which are made fresh or stored for short periods at  $4^{\circ}$ , are added to make the following reagents:

*Reagent B*

Reagent A, 1 ml  
3% BSA, 10  $\mu$ l  
Myokinase, 1.0  $\mu$ g  
Pyruvate kinase, 0.5  $\mu$ g

*Reagent C*

Reagent A, 1 ml  
3% BSA, 10  $\mu$ l  
Myokinase, 2.0  $\mu$ g  
Pyruvate kinase, 1.0  $\mu$ g

*Reagents for Cyclic-GMP Phosphodiesterase Assay*

*Reagent D*

Glycylglycine buffer, 150 mM, pH 8.0  
Magnesium chloride, 6 mM  
Calcium chloride, 30 mM  
Dithiothreitol, 15 mM

As described above, the reagent is 3 times the final concentration and is stable for several months if stored at  $-4^{\circ}$ . Guanosine-5'-monophosphate kinase and 3% BSA are either made fresh or stored for short periods at  $4^{\circ}$ . ATP is either made fresh or stored at  $-4^{\circ}$  for up to 1 year.

On the day of the assay these solutions are added to reagent D to make reagent E as follows:

*Reagent E*

Reagent D, 1 ml  
3% BSA, 10  $\mu$ l  
Guanosine-5'-monophosphate kinase, 20  $\mu$ g  
ATP, 0.1–5  $\mu$ M, depending on desired assay sensitivity

### *Reagent for Assay of ATP*

#### *Reagent F*

Morpholinopropanesulfonic acid (MOPS) buffer, pH 7.8, 10 mM

Magnesium sulfate, 10 mM

0.5% BSA

15 mg/ml purified luciferin-luciferase

Under certain circumstances it may be desirable to increase the concentration of the luciferin-luciferase mixture, or to add additional luciferase.

### *Source and Preparation of the Reagents*

Myokinase (rabbit muscle), pyruvate kinase (rabbit muscle), and guanosine-5'-monophosphate kinase (hog brain) are obtained from Boehringer Mannheim Biochemicals and stored at 4°. The myokinase and pyruvate kinase are received in a suspension of ammonium sulfate. Before use, the suspensions are centrifuged at 1000 g for 10 min, and the resulting precipitates, which contain the enzymes, are dissolved in reagent A to the appropriate concentration. Guanosine-5'-monophosphate kinase, which is in a glycerol solution, is used without further treatment.

A purified luciferin-luciferase mixture is obtained from the instrument division of E.I. DuPont de Nemours & Co. Purified luciferase is obtained from either Sigma Chemical Co. or Calbiochem. All other reagents are obtained from Sigma Chemical Co. or Fisher Scientific.

### *Purification of Cyclic AMP*

Cyclic nucleotides obtained from commercial sources generally contain contaminating nucleotides. Although the concentration of these contaminants is low, they may interfere with the assay. Therefore, the cyclic nucleotides must be purified before use.

Cyclic AMP is purified by precipitating the nucleotide contaminants by the addition of solutions of barium hydroxide and zinc sulfate.<sup>14</sup> To each milliliter of a 100 mM solution of cyclic AMP, adjusted to pH 7.5, is added 0.25 ml of 0.25 M barium hydroxide and 0.25 ml of 0.25 M zinc sulfate. The suspension is mixed and centrifuged, and the resulting supernatant fluid is removed, centrifuged again to remove all traces of the precipitate, and passed through a column (7 × 40 mm) of Dowex 50-X8, 200-400 mesh, hydrogen form. The columns are eluted with water, and 1-ml fractions are

<sup>14</sup> G. Krishna, B. Weiss, and B. B. Brodie, *J. Pharmacol. Exp. Ther.* 163, 379 (1968).

collected. The eluent is monitored by reading the absorbance at 259 nm. The concentration of cyclic AMP is calculated and adjusted to 3 mM on the basis of its molar extinction coefficient ( $E = 15,400$ ).

This solution of cyclic AMP is stable for several months when stored at  $-4^{\circ}$ .

#### Purification of Cyclic GMP

The cyclic GMP is purified by alumina adsorption<sup>15</sup> and ion-exchange column chromatography. One milliliter of a 20 mM cyclic GMP solution in 50 mM Tris HCl, pH 8.0, is placed on a  $7 \times 25$  mm neutral alumina column. The eluent from this milliliter is discarded, and the column is eluted with 50 mM Tris HCl, pH 8.0. The initial milliliter of Tris eluent is discarded. The next 3 ml contain the purified cyclic GMP. One-milliliter portions of these fractions from the alumina column are placed on a  $7 \times 25$  mm column of Dowex 50-X8. The eluent from this milliliter is discarded. The cyclic GMP is then eluted from the column with 2-ml fractions of water. The fractions containing the highest concentration of cyclic GMP, as determined from optical density measurement (252 nm), are combined. The concentration of cyclic GMP is calculated from its molar extinction coefficient ( $E = 13,700$ ) and adjusted to 1 mM. Cyclic GMP purified in this manner typically contains less than 0.005% 5'-GMP and is stable for several months if stored at  $-4^{\circ}$ .

#### Assay Procedure

##### *Cyclic AMP Phosphodiesterase*

This assay is performed in 3 steps. In the first step, the following components are added to a  $6 \times 50$  mm tube: 25  $\mu$ l of tissue sample containing the unknown phosphodiesterase activity, 25  $\mu$ l of reagent B, and 25  $\mu$ l of cyclic AMP.

The reaction sequence is initiated with the addition of cyclic AMP (concentrations from 0.5  $\mu$ M to 1 mM can be used). The samples are incubated for various times at  $37^{\circ}$ , placed in a boiling water bath for 5 min to stop the reaction, and cooled. In the second step, 25  $\mu$ l of reagent C are added to each tube, and the samples are incubated at  $37^{\circ}$  for 1 hr. In the final step, the samples are assayed for ATP by adding 10  $\mu$ l of reagent F to each sample and recording the emitted light.

<sup>15</sup> A. A. White and T. V. Zenser, *Anal. Biochem.* **41**, 372 (1971).



### *Cyclic GMP Phosphodiesterase*

This procedure is also performed in 3 steps. In the first step, the following components are added to a 6 × 50 mm tube: 25  $\mu$ l of tissue sample, 25  $\mu$ l of reagent D, 25  $\mu$ l of cyclic GMP.

The reaction is initiated with the addition of cyclic GMP (concentrations from 0.1  $\mu$ M to 1 mM can be used). The samples are incubated for various times at 37°, placed in boiling water bath for 5 min, and cooled. In the second step, 25  $\mu$ l of reagent E are added to each tube, and the samples are reincubated at 37° for 1 hr. In the final step, ATP concentration is determined by the addition of 10  $\mu$ l of reagent F as described above.

### *Measurement of Generated Light*

The light emission can be quantitated either by scintillation spectrometer or by instruments designed expressly for this purpose, which are available from E. I. Dupont de Nemours, Inc., Aminco-Bowman, Inc., and SAI Technology, Inc.

## **Results**

### *Standard Curves Obtained with These Assay Procedures*

To determine the activity of an unknown sample of phosphodiesterase, assays are run with a standard curve, which consists of varying concentrations of either 5'-AMP (when cyclic-AMP phosphodiesterase is assayed), or of 5'-GMP (when cyclic-GMP phosphodiesterase is assayed). Standards are assayed under incubation conditions identical to those used for the unknown samples. This procedure is designed to account for the presence of interfering enzymes (e.g., nucleotidases) in the sample. In addition, samples of tissue are tested to determine whether they contain nucleotide contaminants, such as ATP, ADP, or 5'-AMP, which can increase the background of the assay. If the sample does contain high concentrations of such nucleotides, the blank can usually be reduced by preincubating the tissue sample to allow nucleotidases in the tissue to convert these nucleotides to the nucleosides, which do not interfere with the assay.

A typical standard curve for 5'-AMP is linear from 10 to 10,000 pmol and is essentially identical to the curve obtained with ATP alone (Fig. 1). This indicates that under the assay conditions all the 5'-AMP is converted to ATP.

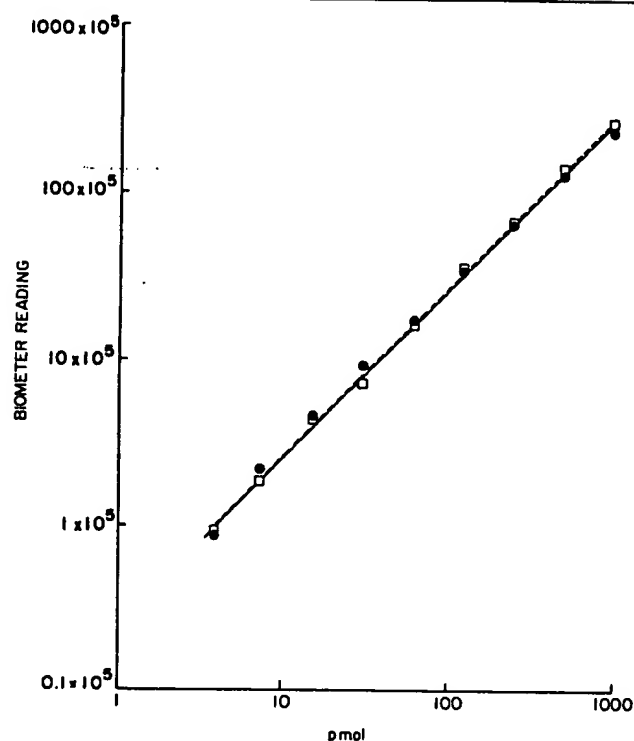


FIG. 1. Conversion of 5'-AMP to ATP. Varying amounts of 5'-AMP (●—●) or ATP (□—□) were incubated for 30 min at 37° under conditions described in the test. The light generated by the ATP in the reaction mixture is expressed in arbitrary units. From B. Weiss, R. Lehne, and S. Strada, *Anal. Biochem.* 45, 222 (1972).

The sensitivity of the assay for 5'-GMP is dependent on the initial concentration of ATP in reagent E (Fig. 2). The lower the initial concentration of ATP, the greater the percentage decrease in ATP with a given concentration of 5'-GMP.

#### *Correlation of Phosphodiesterase Activity with Time of Incubation*

The optimal incubation time for determination of both cyclic AMP and cyclic GMP phosphodiesterase in a given tissue may vary, but in general, product formation is directly related to incubation time for at least 60 min (Figs. 3A and 3B).



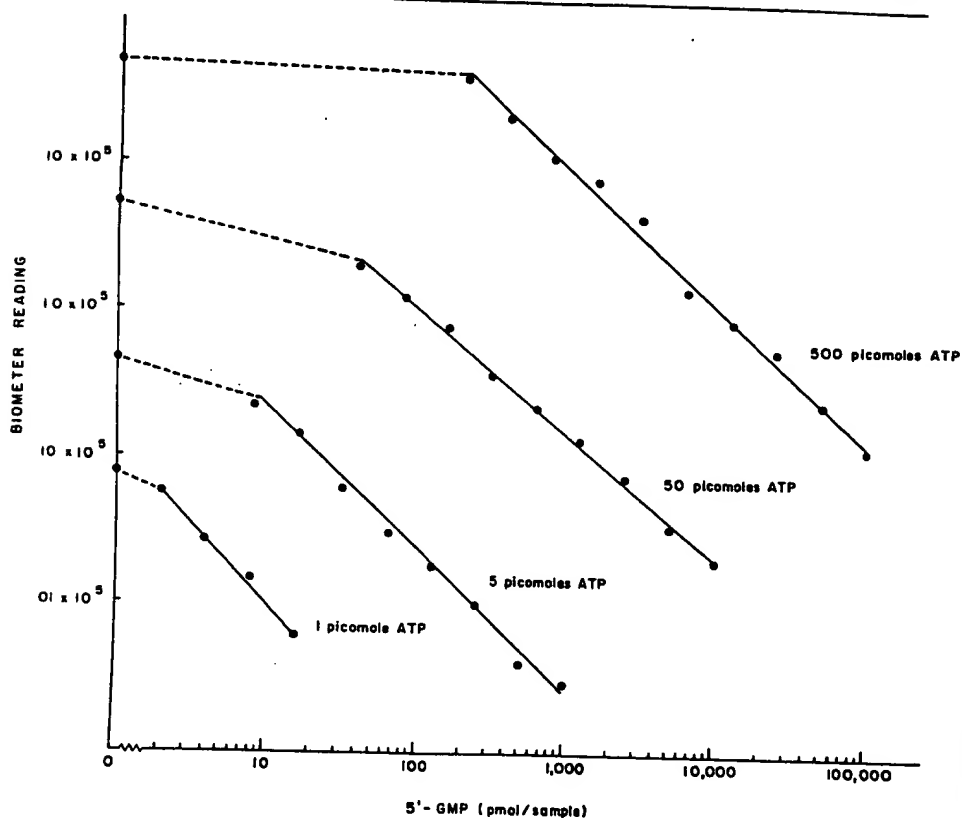
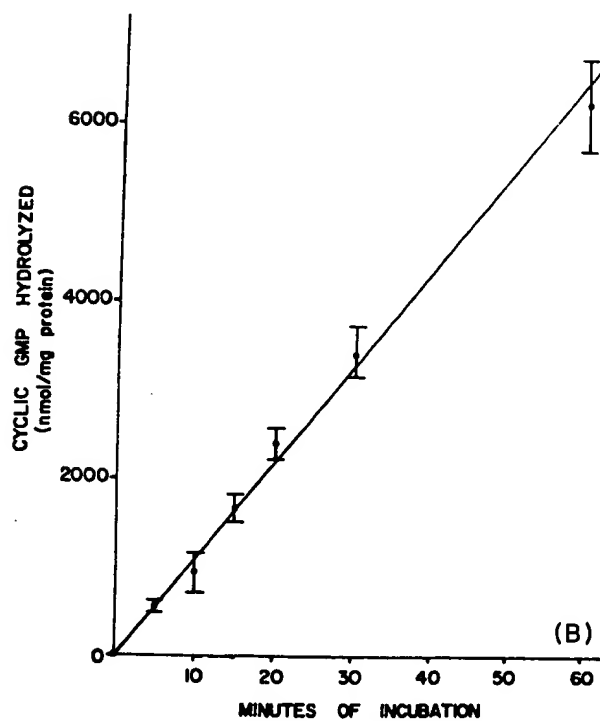
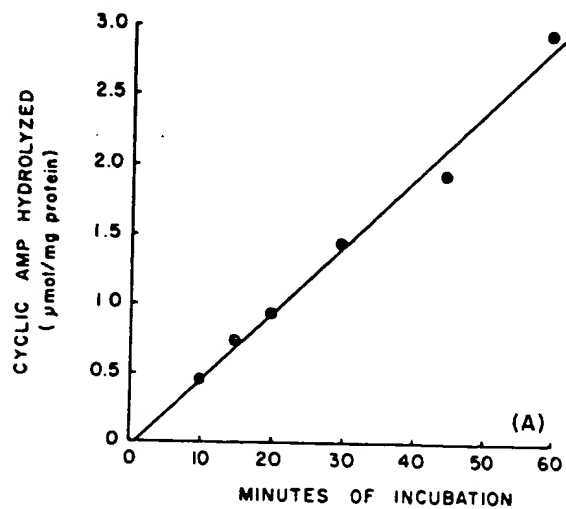


FIG. 2. Sensitivity and linearity of the assay for 5'-GMP. Varying amounts of 5'-GMP and ATP were incubated with reagent E for 60 min at 37°. The amount of ATP that remained is expressed in arbitrary units. Each point represents the mean of five determinations. From R. Fertel and B. Weiss, *Anal. Biochem.* 59, 386 (1974).

FIG. 3. Hydrolysis of cyclic AMP and cyclic GMP as a function of incubation time. (A) Cyclic AMP phosphodiesterase was measured by incubating a homogenate of rat cerebrum equivalent to 1  $\mu$ g of tissue with 0.5 mM cyclic AMP at 37° for varying times. Each point represents the mean of 3 separate assays. From B. Weiss, R. Lehne, and S. Strada, *Anal. Biochem.* 28, 182 (1969). (B) Cyclic GMP phosphodiesterase was measured by incubating a 100,000 g supernatant fraction of rat cerebral homogenate (equivalent to 0.55  $\mu$ g of protein) with 0.2 mM cyclic GMP at 37° for varying times. Activity was determined as described in the text, using 2  $\mu$ M ATP in reagent E. Each point represents the mean of 5 determinations. From R. Fertel and B. Weiss, *Anal. Biochem.* 59, 386 (1974).



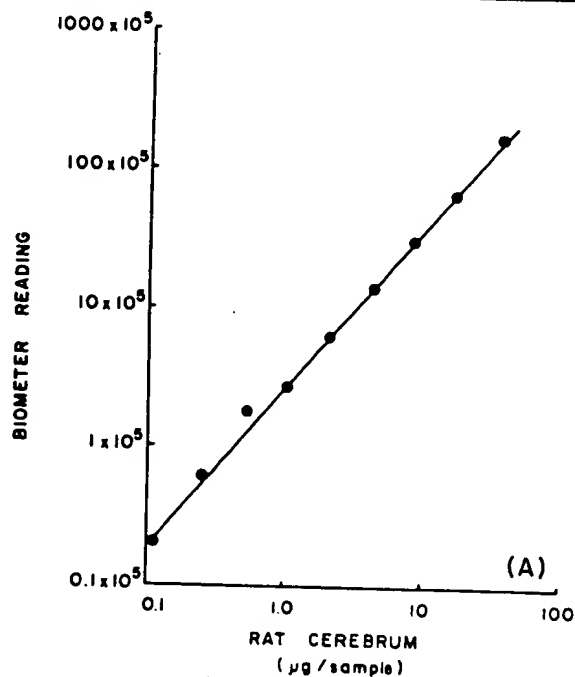


FIG. 4. Hydrolysis of cyclic AMP and cyclic GMP as a function of an increasing concentration of phosphodiesterase. (A) For the cyclic AMP phosphodiesterase, varying concentrations of a homogenate of rat cerebrum were incubated with 0.5 mM cyclic AMP for 60 min at 37° as described in the text. From B. Weiss, R. Lehne, and S. Strada, *Anal. Biochem.* 45, 222 (1972). (B) For cyclic GMP phosphodiesterase, varying concentrations of the 100,000 g supernatant fraction of rat cerebral homogenate was incubated with 0.2 mM cyclic GMP for 60 min at 37°. Enzymic activity was determined as described in the text, using 2  $\mu$ M ATP in reagent E. Each point represents the mean of 5 determinations. From R. Fertel and B. Weiss, *Anal. Biochem.* 59, 386 (1974).

#### *Correlation of Phosphodiesterase Activity with the Amount of Tissue Added*

Both cyclic AMP and cyclic GMP phosphodiesterase activities increase linearly with increasing amounts of tissue (Fig. 4A, 4B). In both assays, the phosphodiesterase activity of less than 100 ng of cerebral protein can be detected, and there is a linear increase in activity over a wide range of tissue concentrations.

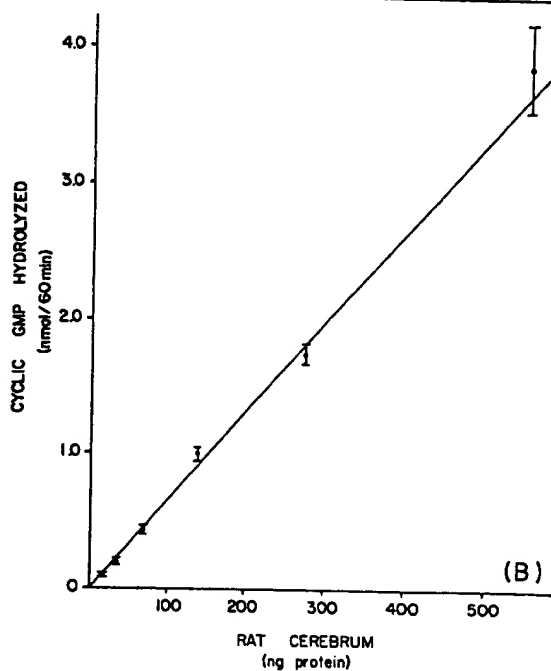


FIG. 4B.

#### Experimental Results Obtained Using These Assay Procedures

The rapidity and low expense of these procedures, coupled with their high sensitivity, permit the analysis of the large number of samples obtained when the multiple forms of phosphodiesterase are studied. (Several hundred assays per day can easily be performed by one investigator.) For example, analysis of the multiple forms of cyclic-AMP phosphodiesterase of rat cerebrum separated by preparative polyacrylamide gel electrophoresis indicate that there are at least four peaks of cyclic-AMP phosphodiesterase activity (Fig. 5).

These assays also have been used to determine tissue and subcellular distributions of phosphodiesterase isoenzymes, their kinetic parameters, and the response of these enzymes to phosphodiesterase inhibition.<sup>14-20</sup>

<sup>16</sup> R. Fertel and B. Weiss, *Mol. Pharmacol.* 12, 678 (1976).

<sup>17</sup> B. Weiss, R. Fertel, R. Figlin, and P. Uzunov, *Mol. Pharmacol.* 10, 615 (1974).

<sup>18</sup> P. Uzunov and B. Weiss, *Biochim. Biophys. Acta* 284, 220 (1972).

<sup>19</sup> P. Uzunov, H. Shein, and B. Weiss, *Neuropharmacology* 13, 377 (1974).

<sup>20</sup> P. Uzunov, H. Shein, and B. Weiss, *Science* 180, 304 (1973).

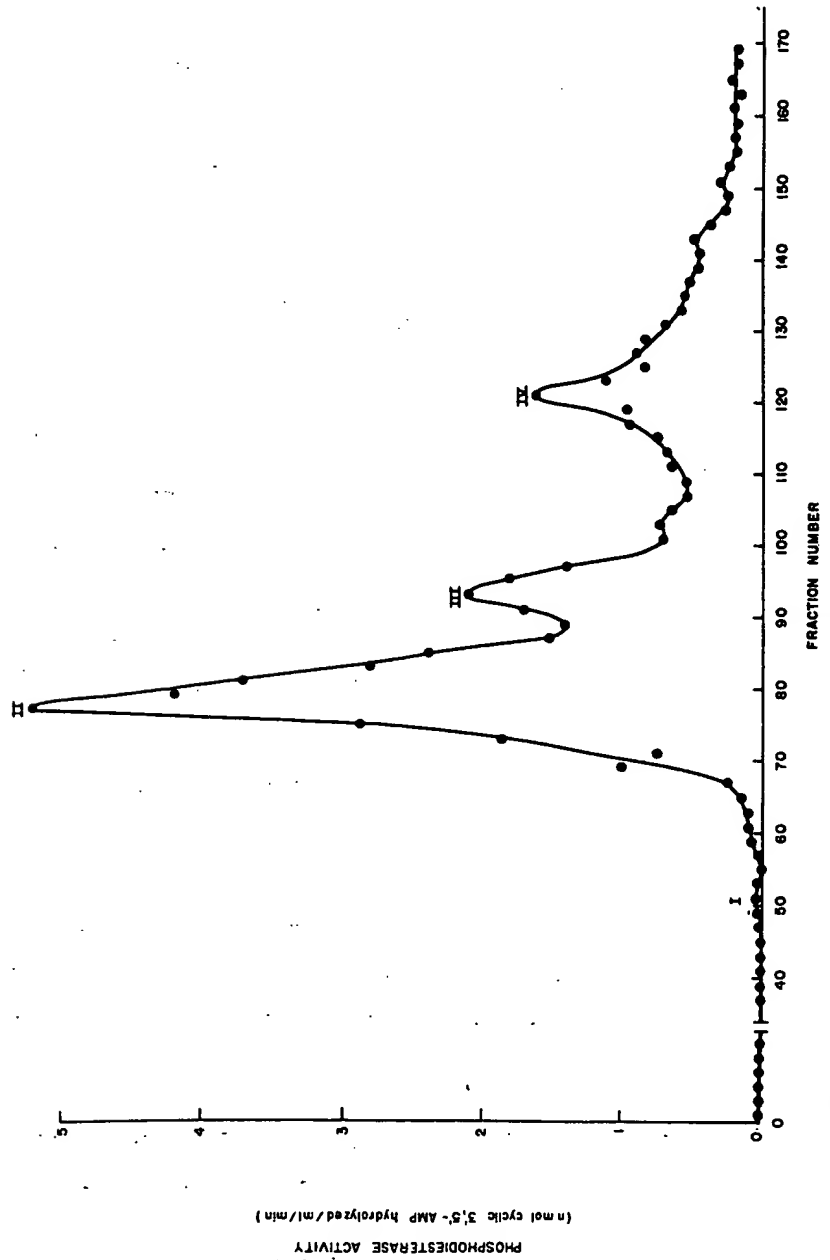


FIG. 5. Rat cerebrum was homogenized in 4 volumes of 0.32 M sucrose, sonicated, and centrifuged at 100,000 g for 60 min. One milliliter of the supernatant fluid was placed on a preparative polyacrylamide gel electrophoresis apparatus. One-milliliter fractions were eluted from the column, and cyclic AMP phosphodiesterase activity was determined as described in the text, using 0.2 mM cyclic AMP as substrate. From B. Weiss, R. Fertel, R. Figin, and P. Uzunov, *Mol. Pharmacol.* 10, 615 (1974).